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NOTES

Listeria monocytogenes Serotype 4b Strains Belonging to Lineages I and III Possess Distinct Molecular Features

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A collection of *Listeria monocytogenes* serotype 4b strains belonging to lineages I and III were examined by PCR and Southern blot analysis using species-, virulence-, and serotype-specific primers and probes. Whereas four serotype 4b lineage I strains reacted in PCR with the serotype 4b-, 4d-, and 4e-specific ORF2110 and virulence-specific *lmo1134* and *lmo2821* primers, all nine serotype 4b lineage III strains were negative by ORF2110 and *lmo1134* primers. In addition, the nine serotype 4b lineage III strains formed two separate groups through their reactions in PCR with virulence-specific *lmo2821* primers. Southern blot analysis using species-specific *lmo0733* and virulence-specific *lmo2821* gene probes largely confirmed the PCR results. These findings indicate that *L. monocytogenes* serotype 4b strains belonging to lineages I and III possess distinct molecular features.

Listeria monocytogenes is a gram-positive bacterium that has the capacity to withstand extreme temperature, pH, and salt concentrations (12, 19). With large amounts of ready-to-eat food products being consumed, it is no surprise that *L. monocytogenes* has emerged as an important source of human foodborne infections (1, 13). As patients with listeriosis often display broad and nonspecific symptoms, such as gastroenteritis, encephalitis, meningitis, septicemia, and abortion, it is vital that rapid, sensitive, and specific tests are available for identification of *L. monocytogenes* to allow appropriate antibiotic therapy. Furthermore, development of typing procedures to trace *L. monocytogenes* strains involved in disease outbreaks will help limit the spread of the disease.

Being one of the first diagnostic techniques developed for *L. monocytogenes*, serotyping exploits the serological reactions of *L. monocytogenes* somatic (O) and flagellar (H) antigens with a series of specific antisera in a slide agglutination format and separates *L. monocytogenes* strains into at least 12 different serotypes (i.e., 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7) (7, 18). Subsequent studies have indicated that serotype classification is clinically relevant, with serotypes 1/2a, 1/2c, 1/2b, and 4b accounting for over 98% of the human listeriosis cases reported (6). Nevertheless, despite offering obvious benefits in the diagnosis and epidemiological investigations of listeriosis, the conventional typing method based on slide agglutination is not performed routinely in clinical diagnostic laboratories, due to its requirement for a whole spectrum of type-specific antisera and due to its apparent lack of reproducibility (17). The development of an enzyme-linked immunosorbent assay (ELISA) platform enables rapid serotyping of *L. monocytogenes*; however, ELISA and agglutination results do not always correspond (15). PCR assays with specificity for various *L. monocytogenes* serotypes have also been described previously (2, 4). Since these PCR assays are not based on genes encoding serotype-specific antigens, they show poor correlation with the agglutination test and are generally incapable of differentiating all serotypes (2, 4).

Apart from the serotyping methods, several genetic methods have been reported for tracking *L. monocytogenes* strains (3, 14, 16, 21, 22). In particular, the application of ribotyping and virulence gene polymorphism analysis led to the establishment of three genetic lineages (or divisions) within *L. monocytogenes*, with lineage I consisting of serotypes 1/2b, 3b, 4b, 4d, and 4e; lineage II of serotypes 1/2a, 1/2c, 3a, and 3c; and lineage III of serotypes 4a and 4c (14, 21, 22). Serotypes that are associated with epidemic human listeriosis often belong to lineage I. Lineage II also contains serotypes frequently associated with human disease, and fewer human listeriosis isolates have been described in lineage III (21, 22).

Sequence analysis of the *prfA* virulence gene cluster has proven useful for phylogenetic and lineage group identification (20). However, the assignment of nine serotype 4b strains (NRRL 33077, NRRL 33092, NRRL 33105, NRRL 33181, NRRL 33183, NRRL 33185, NRRL 33187, NRRL 33190, and NRRL 33191) to lineage III (20) was contradictory to the notion that serotype 4b strains belong to lineage I (14, 21, 22). The purpose of this study was to further investigate *L. monocytogenes* serotype 4b strains belonging to lineages I and III by using the recently reported species-specific *lmo0733* (11), virulence-specific *lmo2821* (recognizing all serotypes except 4a)

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TABLE 1. Examination of *L. monocytogenes* strains by PCR and Southern blot analysis

Strain	Origin	Serotype (lineage)[a]	Reaction with primer in PCR[b]				Southern band(s) (kb)	
			lmo0733	*lmo2821*	*lmo1134*	ORF2110	*lmo0733*	*lmo2821*
EGD-e	Guinea pig	1/2a	+	+	+	−	5.0	5.0
RM3368	Environment	1/2b	+	+	+	−	6.0	5.0
RM3017	Blood	1/2c	+	+	+	−	5.0	5.0
RM3026	Food	3a	+	+	+	−	1.0	4.0, 1.5
RM3845	Hot dog	3b	+	+	+	−	6.0	5.0
RM3159	Human	3c	+	+	+	−	5.0	5.0
ATCC 19114	Human	4a	+	−	−	−	1.5	
RM3177	Human	4b	+	+	+	+	6.0	5.0
RM3030	Cattle	4c	+	+	−	+	1.5	5.0
RM3108	Chicken	4d	+	+	+	−	6.0	5.0
RM2218	Oyster	4e	+	+	+	+	6.0	5.0
NNRL33078	Animal	4b (I)	+	+	+	+	6.0	5.0
NNRL33083	Food	4b (I)	+	+	+	+	6.0	5.0
NNRL33094	Animal	4b (I)	+	+	+	+	6.0	5.0
NNRL33140	Animal	4b (I)	+	+	+	+	6.0	5.0
NNRL33190	Animal	4b (III)	+	+	−	−	1.5	5.0
NNRL33182	Animal	4c (III)	+	+	−	−	1.5	5.0
NNRL33105	Animal	4b (III)	+	+	−	−	1.5	2.0
NNRL33181	Animal	4b (III)	+	+	−	−	1.0	2.0
NNRL33187	Animal	4b (III)	+	+	−	−	1.0	2.0
NNRL33092	Animal	4b (III)	+	+	−	−	1.5	1.5, 1.0
NNRL33183	Animal	4b (III)	+	+	−	−	1.5	1.5, 1.0
NNRL33191	Animal	4b (III)	+	+	−	−	1.5	1.5, 1.0
NNRL33077	Animal	4b (III)	+	−	−	−	1.5	
NNRL33185	Animal	4b (III)	+	−	−	−	1.5	

[a] The serotypes of these strains were determined by agglutination and ELISA (15), and the lineage designations in parentheses were assigned on the basis of their *prfA* virulence gene cluster sequences (20).
[b] +, reacted with primers; −, did not react.

and *lmo1134* (recognizing all serotypes but 4c and 4a) genes (5, 8, 9), and serotype 4b-, 4d-, and 4e-specific ORF2110 (4).

Twenty-five *L. monocytogenes* strains were included (Table 1). Eleven (EGD-e, RM3368, RM3017, RM3026, RM3845, RM3159, ATCC 19114, RM3177, RM3030, RM3108, and RM2218) were chosen to represent the *L. monocytogenes* serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, and 4e, respectively) (15). Of the remaining 14 strains, 9 were classified as serotype 4b and lineage III (NRRL 33077, NRRL 33092, NRRL 33105, NRRL 33181, NRRL 33183, NRRL 33185, NRRL 33187, NRRL 33190, and NRRL 33191), 4 were classified as serotype 4b and lineage I (NRRL 33078, NRRL 33083, NRRL 33094, and NRRL 33140), and 1 was classified as serotype 4c and lineage III (NRRL 330182) (20) (Table 1). In addition, one strain each of *L. grayi* (ATCC 33090), *L. innocua* (ATCC 25400), *L. ivanovii* (ATCC 19119), *L. seeligeri* (ATCC 35967), and *L. welshimeri* (ATCC 43550) was assessed along with the *L. monocytogenes* strains.

L. monocytogenes was initially retrieved from frozen glycerol stock on 5% sheep blood agar plates (TSA II; Becton Dickinson Microbiology Systems, Cockeysville, MD), from which several colonies of each strain were transferred into 50-ml Falcon tubes containing 10 ml of brain heart infusion broth (Difco Laboratories, Detroit, MI) and incubated at 37°C for 18 h with aeration. *L. monocytogenes* brain heart infusion broth cultures (with optical densities at 540 nm of approximately 1.2) were next pelleted by spinning at 3,500 rpm for 15 min. After the supernatant was discarded, the pellets were resuspended in 1 ml of 1× TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0) containing 5 mg lysozyme (Sigma, St. Louis, MO) for 30 min at 37°C. Following addition of SDS and proteinase K, the tubes were incubated at 55°C for 2 h. *L. monocytogenes* genomic DNA was then extracted by phenol-chloroform extraction and ethanol precipitation as previously described (10). The purified DNA was dissolved in 1 × TE, and DNA concentrations were determined at 260/280 nm in a GeneSpec I system (Miraibio, Alameda, CA).

PCR primers from *L. monocytogenes* virulence-specific genes *lmo2821* and *lmo1134* (9), species-specific gene *lmo0733* (11), and serotype 4b-, 4d-, and 4e-specific gene ORF2110 (4, 5) are shown in Table 2. PCR was conducted in a volume of 25 μl consisting of 0.5 U *Taq* DNA polymerase (Fisher Scientific, Houston, TX), 1 × PCR buffer, 50 μM dNTPs, 25 pmol each primer, and 10 ng DNA. Cycling was conducted in a GeneAmp PCR System 2400 (Perkin Elmer, Boston, MA) under the following conditions: 94°C for 2 min once; 94°C for 20 s, 56°C for 20 s, and 72°C for 45 s 30 times; and 72°C for 2 min once. The resultant PCR products were separated by 1% agarose gel in the presence of ethidium bromide (0.5 μg/ml) and visualized under UV light by using a ChemiImager 5500 (BSI, Stafford, TX). Specific bands produced with *lmo0733* and *lmo2821* primers were excised and purified by a Geneclean kit (Qbiogene, Carlsbad, CA) for use as probes in Southern blot analysis.

For Southern hybridization, genomic DNA (20 μg) from each *Listeria* strain was digested with 20 U of restriction enzyme HindIII (Promega, Madison, WI) at 37°C overnight. Approximately 5 μg (per well) from each strain was separated on a 1.0% agarose gel. After denaturation with NaOH and neutralization with Tris-HCl, pH 8.0, the DNA was transferred onto Hybond N+ membranes (Amersham Pharmacia, Piscat-

TABLE 2. Identities of *L. monocytogenes* species-, virulence-, and serotype-specific gene markers

Gene	Putative protein function	Specificity/presence	Forward and reverse primers (5′-3′)	PCR product (bp)	Reference
lmo0733	Transcriptional regulator	Species-specific	CGCAAGAAGAAATTGCCATC TCCGCGTTAGAAAAATTCCA	453	11
lmo2821	Internalin	Present in all serotypes but 4a	TGTAACCCCGCTTACACAGTT TTACGGCTGGATTGTCTGTG	611	9
lmo1134	Regulator	Present in all serotypes but 4a and 4c	ACCCGATAGCAAGGAGGAAC AACTTCTCTCGATACCCATCCA	367	9
ORF2110	Unknown	Specific for serotypes 4b, 4d, and 4e	AGTGGACAATTGATTGGTGAA CATCCATCCCTTACTTTGGAC	597	4

away, NJ) overnight and cross-linked to the membrane by using a GS Gene Linker UV chamber (Bio-Rad, Hercules, CA). The bound DNA was subsequently detected with labeled *lmo2821* and *lmo0733* probes using the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech).

As assessed by PCR, the species-specific *lmo0733* primers recognized all 25 *L. monocytogenes* strains under investigation (Table 1). The virulence-specific *lmo2821* primers detected all *L. monocytogenes* strains except the serotype 4a strain (ATCC 19114) and two serotype 4b lineage III strains (NRRL33077 and NRRL33185) (Table 1). The virulence-specific *lmo1134* primers identified all *L. monocytogenes* strains except for the serotype 4a strain (ATCC 19114), the two 4c strains (RM3030 and NRRL 330182), and the nine serotype 4b lineage III strains (Table 1). In addition to recognizing three known serotype 4b, 4d, and 4e strains (RM3177, RM3108 and RM2218), PCR primers from the serotype 4b-, 4d-, and 4e-specific ORF2110 also reacted with four serotype 4b linage I strains (RM3177, NRRL 33078, NRRL 33083, NRRL 33094, and NRRL 33140) (Table 1).

Southern hybridization analysis using the species-specific *lmo0733* and the virulence-specific *lmo2821* probes further confirmed the PCR results. All 25 *L. monocytogenes* strains were detected with the *lmo0733* probe (Table 1; Fig. 1B), and 22 of the 25 *L. monocytogenes* strains (except serotype 4a strain ATCC 19114 and the two serotype 4b lineage III strains NRRL33077 and NRRL33185) were detected with the *lmo2821* probe (Table 1; Fig. 1A). DNA from the other five *Listeria* species (i.e., *L. grayi* ATCC 33090, *L. innocua* ATCC 25400, *L. ivanovii* ATCC 19119, *L. seeligeri* ATCC 35967, and *L. welshimeri* ATCC 43550) did not hybridize with the *lmo0733* and *lmo2821* probes, verifying the specificity of these gene probes (Fig. 1).

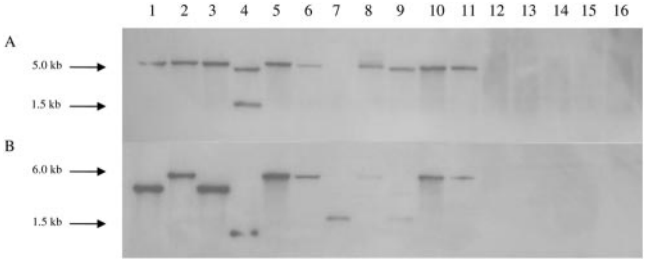

FIG. 1. Southern blot analysis of *Listeria* strains with *lmo2821* (panel A) and *lmo0733* (panel B) gene probes. In both panels, lane 1 contained HindIII-digested genomic DNA of *L. monocytogenes* strain EGD-e (1/2a); lane 2, RM3368 (1/2b); lane 3, RM3017 (1/2c); lane 4, RM3026 (3a); lane 5, RM3845 (3b); lane 6, RM3159 (3c); lane 7, ATCC 19114 (4a); lane 8, RM3177 (4b); lane 9, RM3030 (4c); lane 10, RM3108 (4d); lane 11, RM2218 (4e); lane 12, *L. grayi* ATCC 33090; lane 13, *L. innocua* ATCC 25400; lane 14, *L. ivanovii* ATCC 19119; lane 15, *L. seeligeri* ATCC 35967; and lane 16, *L. welshimeri* ATCC 43550. The numbers on the left are the estimated DNA band sizes (kb).

The species-specific *lmo0733* probe identified a 5.0- or 6.0-kb HindIII band from strains from serotypes 1/2a, 1/2b, 1/2c, 3b, 3c, 4b, 4d, and 4e, as well as all four serotype 4b lineage I strains; however, this probe hybridized with a 1.0- or 1.5-kb band from serotype 3a and 4a strains as well as all nine serotype 4b lineage III strains and the serotype 4c strains (Table 1; Fig. 1B). The virulence-specific *lmo2821* probe detected a 5.0-kb band with serotype 1/2a, 1/2b, 1/2c, 3b, 3c, 4b, 4c, 4d, and 4e strains as well as the four serotype 4b lineage I strains, one serotype 4b lineage III strain, and one serotype 4c strain; and it also hybridized with 4.0-, 2.0-, 1.5-, and/or 1.0-kb bands from the serotype 3a strain and six of the serotype 4b lineage III strains. However, this probe did not react with the serotype 4a strain and two of the serotype 4b lineage III strains (Table 1; Fig. 1A).

The fact that the nine serotype 4b strains belonging to lineage III (NRRL 33077, NRRL 33092, NRRL 33105, NRRL 33181, NRRL 33183, NRRL 33185, NRRL 33187, NRRL 33190, and NRRL 33191) (20) were negative in PCR for serotype 4b-, 4d-, and 4e-specific ORF2110 (4) suggested their non-4b, -4d, or -4e identity. The failure of these nine strains to react with the virulence-specific *lmo1134* primers (previously shown to be present in all but serotypes 4c and 4a) (9) indicated that they may be of serotypes 4c and 4a. This is consistent with their being classified as lineage III on the basis of *prfA* virulence gene cluster sequence analysis (20). Moreover, two of the nine serotype 4b lineage III strains (NRRL33077 and NRRL33185) were negative with the virulence-specific *lmo2821* primers (previously shown to be present in all but serotypes 4a) (5, 9), suggestive of their genetic relationship to serotype 4a strains.

The Southern hybridization results provided additional support that the nine serotype 4b lineage III strains are genetically related to serotype 4a and 4c strains because the species-specific *lmo0733* probe hybridized with 1.5- or 1.0-kb HindIII fragments from their DNA, in contrast with the 6.0-kb fragment that was typical of serotype 4b lineage I strains (Table 1). In addition, two of the serotype 4b lineage III strains (NRRL33077 and NRRL33185) were negative with the viru-

lence-specific *lmo2821* probe (confirming PCR results), typical of serotype 4a strains.

Significant antigen sharing occurs among various *L. monocytogenes* serotypes, with 1/2a and 3c both containing H antigens A and B; 4a, 4b, 4c, 4d, 1/2b, and 3b all having H antigens A, B, and C; 1/2c and 3a both possessing H antigens B and D; and multiple, common O antigens being present in different serotypes (18). Therefore, it can be a challenge to conclusively determine the serotype for some *L. monocytogenes* strains (17). This difficulty in precisely ascertaining *L. monocytogenes* serotypes is one possible reason for the differences in this study between serotype and genotype classifications. Alternatively, it is possible that *L. monocytogenes* serotypes do not always correlate with genetic lineages. Discordant serotype and genotype results for some *L. monocytogenes* strains have been noted previously, and it has been speculated that horizontal gene transfers or point mutations is responsible for causing phenotypic shifts in serotypes (14). Nevertheless, regardless which of these possibilities is more likely, our results demonstrate that *L. monocytogenes* serotype 4b strains belonging to lineages I and III appear to possess distinct molecular features. This emphasizes the need to further identify the genetic determinants responsible for *L. monocytogenes* serotypes to clarify the relationship between serotypes and genetic types. Moreover, future optimization of a multiplex PCR incorporating primers from virulence-specific *lmo1134* and *lmo2821* genes as well as those from ORF2110, ORF2819, *lmo1118*, and *lmo0737* genes (4) may facilitate development of a rapid procedure for simultaneous typing and virulence determination of *L. monocytogenes* bacteria.

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